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The Role of Dietary Nitrate and the Oral Microbiome on Blood Pressure and Vascular tone

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Running title: Dietary nitrate, oral bacteria and vascular tone

Abstract:

There is increasing evidence for the health benefits of dietary nitrates including lowering blood pressure and enhancing cardiovascular health. Although commensal oral bacteria play an important role in converting dietary nitrate to nitrite, very little is known about the potential role of these bacteria in blood pressure regulation and maintenance of vascular tone. The main purpose of this review is to present the current evidence on the involvement of the oral microbiome in mediating the beneficial effects of dietary nitrate on vascular function and to identify sources of inter and intra-individual differences in bacterial composition. A systematic approach was used to identify the relevant articles published on PubMed and Web of Science in English from January 1950 until September 2019 examining the effects of dietary nitrate on oral microbiome composition and association with blood pressure and vascular tone. To date, only a limited number of studies have been conducted, with n=9 in humans and n=3 in animals focusing mainly on blood pressure. In general, elimination of oral bacteria with use of a chlorhexidine based antiseptic mouthwash reduced the conversion of nitrate to nitrite and was accompanied in some studies by an increase in blood pressure in normotensive subjects. In conclusion, our findings suggest that oral bacteria may play an important role in mediating the beneficial effects of nitrate-rich foods on blood pressure. Further human intervention studies assessing the potential effects of dietary nitrate on oral bacteria composition and relationship to real time measures of vascular function are needed, particularly in individuals with hypertension and those at risk of developing cardiovascular diseases.

Key words:

Nitrate, nitrite, nitric oxide, oral microbiome, blood pressure, mouthwash,

Introduction

Cardiovascular diseases (CVDs), including coronary heart disease and stroke, are one of the leading causes of death globally. In 2017, the World Health Organization (WHO) reported that 18 million people had died from CVDs worldwide which represents 31% of deaths[1]. Abnormally raised blood pressure, defined as greater than 140 (systolic)/90 (diastolic) mmHg, is an independent risk factor for CVDs and this silent killer is associated with a three-fold higher risk of having a stroke or developing heart failure[2,3]. High blood pressure affects more than 1 in 4 adults in England, around 12.5 million people. However, the prevalence of hypertension appears to differ between sexes, with 31% reported amongst men and 26% amongst women[4]. Dysfunction of the endothelium, which controls vascular tone and strongly associated with hypertension, is now recognised as an early, but potentially reversible, step in the development of CVDs[5].

The control of vascular function is known to be influenced by dietary factors, with nitrate-rich vegetables considered an important modulator[6,7,8]. This has been demonstrated in many observational and cohort studies which have shown consumption of nitrate and nitrite-rich foods to significantly improve cardiovascular health[9] such as lowering blood pressure[10] in both healthy[11] and hypertensive individuals[12], reducing endothelial dysfunction[13,14 15,16,17] and inflammation[18], protection from ischemia reperfusion injury[19], and improved exercise performance in patients with heart failure[20]. A prospective cohort study has also concluded that an increased adherence to a diet high in nitrate is accompanied by a significant reduction in the risk of suffering both cardiovascular complications and death due to any cause[21]. Clinically, nitrate supplementation or use of nitrate as a medication to increase the bioavailability of nitrite and nitric oxide (NO) can reduce blood pressure[22]. The interest in using dietary nitrates as a treatment for lowering blood pressure is growing but mechanisms underlying the effects are unclear which limits their current application as a dietary treatment for hypertension[22]. Furthermore, there is some evidence to suggest that high dietary nitrate intakes are associated with negative effects on health, which has led to the development of the Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/kg body weight/day and for

nitrite of 0.07 mg/kg body weight/day[23]. The ADI for nitrate is based on the risk of methaemoglobinaemia commonly known as blue baby syndrome, which can occur following high nitrate intake in some babies, and can be fatal [24]. In addition, some epidemiological studies have reported an association between dietary nitrite intake and colorectal cancer. However, the weight of evidence only supports a significant relationship between cancer and red and processed meat[25], with little known about vegetables and drinking water. The nitrate and nitrite within processed meat may be a contributing factor in the association with cancer, although this needs further confirmation.

Humans are naturally colonised by an array of microorganisms, such as commensal or symbiotic communities, whose metabolic activity is important for host physiology and health. Commensal oral bacteria and those residing in the gastrointestinal (GI) tract play an important role in converting dietary nitrate to nitrite and the potent vasodilator NO[26,27,28,29,30,31]. Up to 85% of ingested nitrate is reduced to nitrite by the nitrate-reducing bacteria in the oral cavity[32] raising the salivary nitrite concentration to 1000 times that of plasma[28]. A cohort study conducted in 281 volunteers found that the high abundance of nitrate reducing bacteria was associated with blood pressure in normotensive individuals, although this association was not found in those with hypertension[33]. To date, very little is known about the role of these oral bacteria in the control of vascular function, and the variation in composition that exists between individuals. The aim of this review is to present the current evidence on the potential role of dietary nitrate and the oral microbiome on vascular function including blood pressure and vascular tone. Important determinants of the number and composition of the oral bacteria will also be described. However, the impact of dietary nitrate interventions on vascular function only will not be specifically addressed in this instance due to the large number of review articles which already exist in this research area[13,14 15,16,17]. Before presentation of the methodology and results of the literature review, we provide a general overview of dietary nitrate sources, the pathways for the conversion of dietary nitrate and nitrite to NO, location and type of nitrate-reducing bacteria in the oral cavity and their potential role in regulating vascular tone.

Nitrate, nitrite and nitric oxide sources and nitric oxide pathway

NO, the most effective form of nitrate, was first recognised in 1998 as an important signalling molecule in the cardiovascular system[34]. NO plays a significant role in virtually all organs in the body, and higher circulating concentrations are associated with a lower CVD risk[35]. In additional to the dietary (exogenous) sources of nitrate and nitrite which leads to the production of nitrite, and subsequently NO, via the oral bacteria, the body can also derive NO endogenously (figure1). The endogenous pathway can occur in a number of different tissues in the body using three forms of NO synthase (NOS) enzyme, neuronal (nNOS), endothelial (eNOS) and inducible NOS (iNOS). eNOS was initially discovered in endothelial cells and is important in modulating vascular tone and upholding endothelial integrity. However, eNOS can also be expressed in various tissues and requires the presence of oxygen, calcium and calmodulin to be activated[36]. Within the endothelium, L-arginine undergoes a 5-electron oxygen dependent oxidation to produce NO and L-citrulline, catalysed by the synthase enzymes. Five cofactors required by the NOS enzymes are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH4), reduced nicotinamide-adenine dinucleotide phosphate (NADPH) and heme iron[28]. Once produced in the endothelial cell, NO rapidly diffuses to the underlying smooth muscle layer where it mediates blood vessel vasodilation. Any NO remaining in the circulation is rapidly converted to nitrate by oxyhaemoglobin or superoxide before it enters the enterosalivary pathway. Therefore, the NO produced has a relatively short half-life in the order of seconds to minutes[37].

Nitrate metabolism, enterosalivary circulation and gastrointestinal tract

High levels of inorganic nitrate are found in vegetables (such as beetroot and spinach) as well as drinking water, and these dietary sources accounts for 80% of the daily intake. In contrast, the intake of dietary nitrite is very low, being approximately 100 times lower[38] than that of nitrate [39]. Although the process of re-circulation of nitrates in the body has been known since 1970s, the

importance of the oral nitrate-reducing bacteria in the enterosalivary circulation has only recently been recognised[27] (Figure 2). The key role these bacteria play in nitrate reduction was supported by a previous human study in which a significant correlation was found between high abundance of oral nitrate-reducing bacteria and nitrite level in saliva[40]. Nitrate secretion from the salivary glands leads to a 10 fold rise in salivary nitrate levels[41] and this nitrate enriched saliva appears to be a supportive environment for the growth of the oral bacteria particularly the nitrate-reducing bacteria on the tongue[42]. These bacteria are mostly facultative anaerobes which use nitrate as an alternative electron acceptor for their respiration[43]. A symbiotic relationship therefore exists between the oral commensal bacteria in which they receive nitrate from the host for their own respiration and in return produce nitrites required by the host[42]. This relationship is particularly important for nitrite bioavailability since humans are unable to complete this process independent of the nitrate-reducing bacteria, with 80% of nitrates swallowed and present in the stomach produced by the oral commensals[44]. Once in the stomach, contact with the gastric acidity leads to the protonation of nitrites to form nitrous acid (HNO_2), which then decomposes into not only NO but also several other nitrogen oxides[45] which have localised benefits on maintaining the gastric mucosa layer[46] and enhancing mucosal blood flow[45] which increases the thickness of the mucosal layer[47]. This process is referred to as non-enzymatic conversion which does not require bacteria. However, the presence of *Helicobacter pylori* can contribute to a more acidic environment within the stomach and increase non-enzymatic conversion[48]. Residual nitrates and nitrites are then absorbed in the small intestine with the half-life of circulating nitrate in the blood stream of around 5-6 hours[49]. In contrast, plasma nitrite concentrations start to increase within 15 minutes of nitrate ingestion and reach a peak level in 2 hours[50]. A large portion, approximately 70-75% of the plasma nitrate, is excreted in the urine whereas the remaining 25% is stored in the salivary gland and then recycled in the enterosalivary pathway[51].

The role of the nitrate-reducing bacteria can persist past the oral cavity as most of these bacteria move into the stomach with both swallowed food and saliva. Limited studies have

investigated the existence of these bacteria in the stomach and have confirmed that the gastric acidity is not a germ-free environment[52]. Although the gastric pH is below 5, some bacteria species can tolerate the stomach acidity, with a culture based study reporting *Clostridium* spp, *Veillonella* spp and *Lactobacillus* spp as the most predominant gastric species[53], with *Veillonella* spp identified as the most abundant nitrate reducing bacteria[43]. There are many factors that can influence gastric acidity such as inflammation and long-term use of proton pump inhibitors. The pH level has been found to have a positive impact on nitrate and nitrite concentration in the gastric juice. In a study conducted in 99 patents with dyspepsia, results showed that when the pH level of gastric mucosal surface increased there was a comparable increase in both nitrate and nitrite concentrations. Findings from another study conducted in participants with achlorhydria , in which gastric pH ranged from 6-8, reported three genera of nitrate reducing bacteria: *Streptococci* and *Neisseriae* to be responsible for the nitrite accumulation in the gastric secretions[54].

The small intestine and colon contain many different species of bacteria including both facultative and obligate anaerobes which are involved in the bioconversion of nitrite to NO, although they are not necessarily the same as the nitrate reducing bacteria found in the oral cavity[55]. A study conducted in germ-free and normal rats has shown that NO can be produced by the bacteria resident in the small intestine of normal rats, but not in germ free rats[56]. Furthermore, two studies have identified *Lactobacilli, Bifidobacteria*[56], *Escherichia coli and Shigella* as the predominant nitrate reducing bacteria in the large intestine[57]. However, an *in-vitro* study which used pure strains of gut bacteria incubated in agar media with nitrate then nitrite, found that in the presence of nitrite, both *Bifidobacterial* and *Lactobacilli* generated large amounts of NO, up to 5000 parts per billion (ppb), but only approximately 35 ppb of nitrate[58]. Interestingly, Sobko et al reported that the NO formed was being utilised by *Escherichia coli* and *Staphylococcus aureus* [46]. These authors speculated that these gut bacteria may consume NO in order to help adapt to their environment in this *in vitro* experiment. Therefore, it appears that the presence of NO and other nitrate metabolites in the large intestine may be dependent on the relevant abundance of these bacteria species and their

production and utilisation of NO [59]. Localised effects of the NO could include altering blood flow which could potentially increase the uptake of nitrate and nitrite in the proximal small intestine where the majority are absorbed[44]. However, the NO level in the GI tract could also be influenced by other factors such as pH level, inflammation, oxygen tension and the level of dietary nitrate intake of an individual. Further studies are need to determine the direct effects of nitrate and nitrite on gut bacteria composition and nitrate metabolism.

Bacterial nitrate reduction in the oral cavity, composition and location

A continuous flow of saliva, specialized mucosal surfaces and teeth in the human oral cavity provide a unique microbial habitat for bacteria. Most of these bacteria are found on the dorsum (surface) of the tongue and around the teeth where a wash of 1 ml of saliva can contain up to 10^7 – 10^8 microorganisms[44]. However, only 700 species have currently been identified[44]. The majority of these bacteria shelter in the gingival crevices between teeth which represents a conducive anaerobic environment. Here, the gingival crevicular fluid bathes the bacteria within a nutritionally rich medium supporting their proliferation[60]. In contrast, the smoother surfaces of teeth have much lower levels of bacteria due to the forces that act on these areas during eating and drinking. However, the nitrate-reducing bacteria are found predominately on the rear dorsum of tongue, with a higher proportion of gram-negative bacteria found within the papillae of the tongue compared to the surface. Some studies have identified the genus and species of these bacteria that can produce nitrate reductases and nitrite reductases that aid in the production of nitric oxides. These include: *Veillonella atypical Veillonella dispar, Actinomyces eslumdii, A. odontolyticus, Staphylococcus epidermids, Neisseria flarescens, Haemophilus, Porphyromonas, Rothia mucilaginosa, Rothia dentocarisa, Prevotella and Leptotrichia*[42,43]. The two major groups of oral nitrate-reducing bacteria are the strict anaerobes such as *Veillonella atypica* and *Veillonella dispar* and the facultative anaerobes such as *Actinomyces odontolyticus* and *Rothia mucilaginosa*[42]. Facultative anaerobes are mostly prevalent on the surface of the tongue, with a study stratifying participants

according to oral nitrate reduction capacity observing a higher abundance of *Streptococcus, Granulicatella, Prevotella, Neisseria, and Haemophilus* on the posterior surface of the tongue compared to *Actinomyces*[43]. Interestingly, although lower in prevalence, *Actinomyces* have been reported to be more efficient reducers of dietary nitrates under anaerobic conditions.

Mechanisms by which bacteria may convert nitrate to nitrite

The three mechanisms through which nitrates are converted to nitrites and other components by bacteria are denitrification, assimilation and dissimilation. The first process, denitrification, occurs in the oral cavity under aerobic conditions[61] and is also called the respiratory nitrate reduction process. During microbial respiration, oxygen is replaced by nitrogen oxides as terminal electron acceptors and ultimately reduces nitrate to nitrous oxide or free nitrogen[62]. Most of the bacteria which have genes for respiratory nitrate reductases (*nirS* and *nirK*) prefer aerobic conditions[63] such as *Rothia spp* and *Neisseriae spp*. However, some denitrification species of bacteria also reside in anaerobic conditions[44] such as *Veillonella*. The specialised surface of the tongue dorsum therefore represents a microaerophilic environment which allows denitrification to occur under both aerobic and anaerobic conditions. In the oral cavity, nitrite (NO_2) is initially formed from salivary nitrate (NO_3) by some oral bacteria such as *Actinomyces*[43] that are considered to possess the nitrate reductase enzyme (*nar*) and further converts nitrite to NO through either enzymatic (*nir*) or non-enzymatic denitrification. The latter process is a well-established step in the gastric environment of the stomach. NO is then converted to nitrous oxide (N_2O) by nitric oxide reductase (*nor*) and finally to nitrogen (N_2) by nitrous oxide reductase (*nos*). The nitrogen oxides and enzymes that participate in the process of denitrification are as follows:

$$NO_3 \xrightarrow{nar} NO_2 \xrightarrow{nir} NO \xrightarrow{nor} N_2O \xrightarrow{nos} N_2$$

In the second pathway known as dissimilation, nitrate is reduced to ammonia (NH_4+) by periplasmic nitrate reductase (*nap*), with the intermediate product being nitrite[64]. This two-step process is strictly anaerobic and occurs in the human gut by the facultative anaerobes[55].

$$NO_3 \xrightarrow{nap} NO_2 \xrightarrow{nrf} NH_4+$$

Assimilation, which occurs predominantly in plants, water and soil[65], is the third pathway. Similar to denitrification, the conversion of nitrate to ammonia occurs but during this pathway, the enzyme cytoplasmic nitrate reductase (*nas*) is used[65]. In this biosynthetic anabolic pathway, nitrite is further reduced to ammonia, which can then undergo ammonium assimilation by incorporating the amino acid glutamine[44]. The assimilation and dissimilation processes are therefore important in the utilization of nitrates**.** Nitrifying bacteria (including *Nitrobacter, Nitrococcus* and *Nitrosomonas*)[66] are responsible for the dissimilation and ammonification of nitrates and oxidises ammonium salts and nitrites to nitrates in a process called nitrification. It has been hypothesised that this process might happen in the gut, but to date, this has not been described[67]**.**

In humans, nitrate reduction seems to occur either directly, such as in assimilatory nitrate reduction, or during a series of reactions during respiratory nitrate reduction. Notably, the latter process needs more than one enzyme for further reduction which is mediated by the bacterial communities[44]. This suggests that nitrate reducing capacity of nitrate-reducing bacteria is related to the bacterial species, cellular location of enzymes and environmental conditions such as oxygen level. Therefore, dissimulation would occur more in the gut and denitrification in the oral cavity[67]. Although the role of oral bacteria in mediating the beneficial effect of nitrate on vascular function is poorly understood, this review aims to address this knowledge gap by focussing on studies that used antibacterial mouthwash and toothpaste to determine the importance of the presence of oral microbiome on blood pressure and vascular tone.

Methods

A systematic approach was used to identify the relevant human and animal studies which investigated the role of dietary nitrate and the oral microbiome on blood pressure. PubMed and Web of Science were used for the literature search which included all relevant articles published in English from January 1950 until September 2019. There were three stages in the selection process. The combinations of the key terms used in the search strategy were as follows: ("Nitrate" OR "Nitrite" OR "Nitric Oxide") AND ("Oral Bacteria" OR "Oral Microbiom" OR "Nitrate-Reducing Bacteria") AND ("Blood Pressure" OR "Hypertension" OR "Cardiovascular" OR "Vascular Function") AND ("Mouth Wash" OR "Antiseptic" OR "Antibacterial"). The titles and abstracts of the identified papers were screened by one member of the review team (HA) who identified potentially relevant papers. This review was restricted to animal studies and human studies which used antibacterial mouthwash or toothpaste to determine the effects on oral nitrate reduction on blood pressure and vascular tone. Only published peer-reviewed literature was considered and 'grey' literature such as dissertations, conference proceedings, reports, letters to editors and other non-peer-reviewed research, was excluded. After duplicates were removed, the abstract and full papers were screened for eligibility. In addition, a hand-search of the bibliographies of the articles found from the electronic database searches was also conducted. An overview of the literature search is shown in Figure 3.

The quality of the included human RCTs and animal studies were assessed for the risk of bias using the Cochrane risk of bias tool[68] for human studies and SYRCLE's tool[69] for animal studies.

Results and Discussion

The systematic search identified 160 publications. Of these, 11 relevant publications were included, with 9 describing studies conducted in humans and 3 in animals. The risk of bias assessment summaries for each study are presented in Supplementary Tables 1 and 2, respectively. Animal studies will be discussed before studies including human participants. This will be followed by

discussion of the non-modifiable and modifiable factors affecting intra-individual variability in number and composition of oral bacteria, with potential mechanisms of action.

Animal studies

Of the 14 animal studies which have investigated the effect of nitrate on blood pressure, only 3 studies have determined whether oral bacteria are important in mediating the improvements in blood pressure and endothelial function (Table 2). Formation of bioactive NO takes place within the gastric environment of the stomach as a result of the enterosalivary circulation of nitrate, as well as systemically in the blood vessels. In 2009, Petersson and his colleagues[70] reported daily mouthwash treatment for 7 days in rats to attenuate both the gastroprotection provided by NO and the diastolic blood pressure lowering effect of sodium nitrate. A similar pattern was also evident for the mean arterial pressure in the rats treated with mouthwash and nitrate, but the lack of an effect in the rats treated with mouthwash and nitrite suggested that oral bacteria play an important role in the metabolism of nitrate to NO and mediated vasodilation. Furthermore, these rats also had reduced oral bacteria suggesting that nitrite could bypass the reduction step by the oral bacteria and was being reduced in the circulation or within endothelial cells to NO, or via effects on the formation of the intermediate nitrosothiols[70]. However, dietary nitrite intake is generally lower than that of nitrate, and the half-life in plasma shorter (seconds versus hours) which suggests that even if nitrite directly stimulates NO signalling, the quantity and kinetics of nitrite versus nitrate indicates that the critical aspect of this mechanism is the reduction of nitrate. Therefore, the role that dietary nitrite plays in blood pressure lowering may be more limited relative to nitrate.

In agreement, Hyde et al[29] also reported a significant reduction in diastolic blood pressure and increase in plasma nitrite concentrations following the addition of sodium nitrate to drinking water in male Wistar rats. However, in this study, mouthwash treatment was unable to diminish the blood pressure lowering effects of the nitrate supplementation. The authors speculated that the direct application of the chlorhexidine-based mouthwash (Vedco, St. Joseph, MO) to the tongue

surface using a swab might not have enabled sufficient time for the mouthwash to exert its full extent on the bacteria relative to mouthspray[29]. A novel aspect of this longer-term supplementation study was the focus on the changes in the microbiota composition on the rat tongue in response to the treatments. Compared with baseline, there was a greater relative abundance of nitrate reducing bacteria (*Haemophilus spp* and *Steptococcus spp*) after 6 days of sodium nitrate consumption, and of these *Haemophilus parainfluenzae* has also been identified as 1 of 14 species contributing to nitrate reduction in the oral cavity of healthy adults. Co-supplementation of mouthwash with nitrate was found to increase the diversity of the oral bacteria present relative to nitrate intake only, with increases found in the low abundance taxa such as Enterobacteriaceae, *Corynebacterium*, and *Morganella*. Therefore, the use of mouthwash appeared to disturb the oral microbiome by reducing the abundance of the normally dominant taxa but not completely to impact nitrate reduction. These findings suggest that the lower abundance taxa which were evident after mouthwash treatment may be functionally important in the bioactivation of dietary nitrate. However, the authors did caution against translating these findings on the oral bacteria composition to humans since the oral human microbiome has been shown to be more diverse and of a differing composition compared with the rat[29].

The impact of mouthwash on chronic changes in blood pressure in response to nitrate or nitrite supplementation was further examined by Pinheiro et al[71] in both control and hypertensive rats. After 4 weeks, significant reductions in mean arterial pressure and systolic blood pressure were evident in both the nitrate and nitrite groups, with concordant increases found in circulating plasma nitrate and nitrite levels. Interestingly, co-supplementation with mouthwash attenuated the rise in plasma nitrite levels by 25-30% in both groups but was only found to blunt the blood pressure lowering effect of nitrate, with little impact found on blood pressure in the mouthwash and nitrite group. In agreement with Petersson et al[70], these findings suggested that anti-hypertensive effects of nitrite were potentially occurring via non-enzymatic reactions within the gastric environment after swallowing this ion independently of the enterosalivary pathway and potentially via non-enzymatic

reactions within the gastric environment after swallowing this anion. Analysis of the endogenously produced vasodilatory compound S-nitrosothiol and levels of vascular nitrosylation revealed mouthwash to reduce nitrosylation responses to nitrate only, leading the authors to speculate that S-nitrosylation was an important mediator of the blood pressure lowering effects of both nitrate and nitrite[70,71]. Studies have also reported that the foods consumed with dietary nitrites, such as conjugated fatty acids, are also a target of nitrating species in the stomach leading to the formation of nitro-fatty acids (such as nitro-conjugated linoleic acid). These electrophiles have been shown to have anti-hypertensive effects independent of S-nitrosothiols suggesting that they may also play a role in mediating the effects of nitrate and nitrite on blood pressure [72]. Antiseptic mouthwash was proposed to attenuate the beneficial effects of dietary nitrate intake on blood pressure by reducing the amount of nitrite formation by the oral bacteria and therefore reaching the stomach, inhibiting gastric formation of S-nitrosothiols. However, the positive benefits on blood pressure of raised S-nitrosothiols was only found in the antihypertensive rats, supporting previous observations in both animals and humans that raised blood pressures often show a greater sensitivity to the anti-hypertensive effects of medication and/or dietary modification.

Studies performed in animals may provide useful insights into the mechanisms underlying the effects of oral bacteria in the bioactivation of nitrate. However, findings in rats and mice need to be interpreted with caution due to differences in physiology and dependence on nitrate as a source of NO between organisms. In contrast to humans, rats and mice do not recirculate nitrate in saliva[73] and so salivary nitrate concentrations never exceed those levels found in plasma[74] and they also have other nitrate reducing mechanisms that may work in tandem with nitrate reduction by the oral bacteria to control nitrite and NO level[73].

Human Studies

The publications describing the human studies were divided into those which examined 1) the association between oral bacteria with nitrate/nitrite levels and/or blood pressure (n=5; Table 2) and

2) the combined effects of nitrate ingestion and oral bacteria on nitrate/nitrite levels and/or blood pressure (n=4; Table 3). The role of the oral bacteria in mediating systemic nitrite production after nitrate intake has been primarily investigated with the use of an antiseptic mouthwash to remove the bacteria prior to the measurement of the outcomes of interest. The type of mouthwash has been shown to be important, with the strong antibacterial chlorhexidine-based mouthwash (Corsodyl) found to be more effective at reducing *Veillonella dispar* (nitrate reducing bacteria) in the oral cavity than Listerine (mixture of essential oils), Isodine and Cepacol (antibacterial) in healthy adults[75]. In support of these findings, gargling with 10 ml of chlorohexidine mouthwash (Corsodyl) twice for 1 min was also found to reduce the bacterial count of nitrate reducing bacteria by approximately 80% and virtually abolish the oral nitrate reducing capacity compared with no mouthwash in healthy subjects[27]. Although nitrate accumulated in saliva after ingestion of sodium nitrate in both studies, a significant reduction in the conversion of salivary nitrate to nitrite after mouthwash was associated with 30% lower plasma nitrate concentrations at 3 h post-ingestion, compared with no prior use of mouthwash. In contrast, a randomised cross-over study found an antibacterial toothpaste to have no effect on salivary or plasma nitrate concentrations in 16 women after consuming 400 mg of nitrate before brushing their teeth with antibacterial toothpaste (0.3% triclosan) or toothpaste containing no antibacterial agent[76]. The lack of an effect observed with the antibacterial toothpaste may reflect either the lower prevalence of the nitrate reducing bacteria on the surface of the teeth, relative to the tongue, or the less efficient removal of the bacteria sheltering within the gingival crevices between the teeth compared with mouthwash.

Four studies have determined the impact of mouthwash on changes in oral nitrate reducing capacity and blood pressure (Table 2). Compared with no mouthwash, Kapil et al[41] reported that using 0.2% chlorhexidine twice daily for 7 days significantly increased systolic and diastolic blood pressure measured using 3 different techniques (clinic, ambulatory and home measurements) by approximately 3 and 2 mmHg respectively in 19 healthy normotensive subjects. Interestingly, the effects of mouthwash treatment on blood pressure was evident after only a single use of the

chlorhexidine mouthwash and was maintained for the following 6 days. The rise in blood pressure was significantly correlated with the significant reduction in plasma nitrite levels, with only a trend for a relationship with the salivary nitrite, highlighting the potential importance of the oral nitrate-reducing bacteria in blood pressure modulation.

In 15 subjects treated with anti-hypertensive medication, the attenuation found in oral nitrate reducing capacity after daily use of chlorhexidine mouthwash for 3 days was associated with an increase in systolic blood pressure of 2.3 mmHg, but only a trend for a decrease in plasma nitrite concentrations compared with the control (tap water)[77]. The lack of a significant effect on the plasma nitrite response relative to Kapil et al[41] was thought to be due to the study visit being performed 12 h after prior use of the mouthwash treatment or related to the age or medication use of the hypertensive participants. In order to determine the mechanism underlying the effects of dietary nitrate intake on blood pressure, plasma cGMP, a mediator of NO-dependant smooth muscle relaxation in the endothelium and a good marker of NO production, can be measured. Although increases in plasma nitrite and cGMP after dietary nitrate intake have been previously associated with blood pressure lowering, no effects were evident on cGMP concentrations after 3 days of using mouthwash. This may be related to the lack of a nitrate challenge on the study visit (which provides an important source of NO under hypoxic conditions) but could also suggest that dietary nitrate may impact on vascular tone via direct effects on smooth muscle function.

In contrast to these two studies, Tribble et al [78]reported use of chlorhexidine mouthwash twice daily for 7 days to be associated with a highly variable effect on clinic systolic blood pressure (an increase of at least 5 mmHg found in n=9 subjects whereas a decrease was observed in n=4) in an orally healthy cohort. Post-hoc data analysis revealed the inclusion of tongue cleaning as part of the daily dental hygiene routine to play a significant role in the responses observed both on blood pressure and the diversity of the oral bacteria at baseline and during the study. Specifically, regular tongue cleaning was associated with a greater ability to reduce nitrite to NO whereas the lack of tongue cleaning resulted in an oral microbiome composition which favoured conversion of nitrite to

ammonia and not NO. The authors speculated the use of chlorhexidine mouthwash was having a chemo-stimulatory effect on the oral bacteria, with the temporary loss of bacterial numbers proposed to stimulate a rapid population recovery and increase in bacterial nitrate reductase activity. However, these effects may also reflect a protective upregulation of the nitrate, nitrite and NO regulating mechanisms in the microbiota suddenly detached from their biofilms during tongue cleaning and warrants further investigation.

In a cross-over study, treatment with chlorhexidine (0.2%) for 3 days was shown to have no effect on clinic or 24 h ambulatory blood pressure in 17 young females compared with a placebo mouthwash [79]. Although a reduction in salivary nitrite and oral nitrate reducing capacity was found after the antibacterial mouthwash, comparable changes were not evident in either the plasma or urine samples collected. The lack of effects observed relative to other studies may reflect the short intervention time with the mouthwash treatments or inclusion of female participants only. Based on a previous study conducted by the same research group in athletes, they speculated that cross-talk may exist between the enterosalivary nitrate-nitrite-NO pathway and eNOS, with a greater intake of dietary nitrate associated with a lower eNOS activity. However, whether a reduction in nitrate-nitrite-NO with antibacterial mouthwash leads to an upregulation in eNOS is yet to be established.

In the studies presented in Table 3, measures of blood pressure have been related to salivary and plasma nitrate/nitrite levels following nitrate intake and use of mouthwash. In agreement with previous findings, Woessner et al [30] found antibacterial mouthwashes to attenuate postprandial salivary and plasma nitrite concentrations following dietary nitrate intake (concentrated beetroot juice) compared with the weaker antiseptic mouthwash and control. Although changes in clinic systolic blood pressure 0-3 h after the treatments were not related to plasma/salivary nitrite or nitrate levels, systolic blood pressure at 4 h was 2-5 mmHg higher after Chlorhexidine and Cepacol mouthwashes compared with control and Listerine mouthwash. These findings potentially suggest an important role of the nitrate-nitrite-NO enterosalivary pathway, but should be interpreted with caution due to the small sample size, inclusion of male subjects only and the short duration of the

study visit relative to the expected peak in plasma nitrite concentrations (approximately 3 h). Furthermore, these findings may have been influenced by the large inter-individual variability observed in blood pressure responses following the mouthwash treatments.

In the study of McDonagh and co-workers [80], consumption of 2 x 70 ml shots of concentrated beetroot juice and daily use of strong or weak antibacterial mouthwash for 6 days were found to have limited effects on baseline blood pressure and salivary and plasma nitrate/nitrite levels compared with the control (water). However, differences were evident 2-4 h after drinking the beetroot juice, with the rise in plasma nitrite found to be attenuated after use of the strong and weak mouthwash for 6 days. These changes were associated with a reduced oral nitrate reducing capacity after the strong mouthwash, with lower nitrite levels compared with both the weak and placebo mouthwashes. Although changes in resting measures of blood pressure (supine and seated) and pulse wave analysis (arterial stiffness) after the juice were not influenced by the strength of the mouthwash used, differences were evident in blood pressure during low-intensity activity on the treadmill. In particular, there was a greater increase in systolic blood pressure and mean arterial pressure after rinsing with the strong (Chlorhexidine) compared with the control (water) mouthwash. The lack of effect on arterial stiffness even in the presence of lower salivary and plasma nitrite levels after the strong mouthwash indicates that either the availability of NO was not altered sufficiently over the 4 h acute test period in these young active participants or that their higher physical active level may have masked any effects of the mouthwash on the vascular function measures. However, this is one of the only studies to incorporate a measure of blood vessel elasticity to determine the role of oral bacteria in mediating the beneficial effects of beetroot juice on vascular function, and so further studies are needed in which to compare these findings and determine the underlying mechanisms.

As highlighted in the human studies, oral bacteria composition appears to vary between individuals, with both non-modifiable (e.g. age, sex, genetics and tongue physiology) and modifiable (e.g. diet, health conditions, life style and dental hygiene routine) factors considered to

impact on the abundance and prevalence of nitrate reducing bacteria in the oral cavity. These factors are important to consider during interpretation of the study findings and for informing the design of future studies exploring the role of oral nitrate reducing bacteria on the regulation of vascular function. The following section summarises the main factors identified from the human studies.

Intra-individual variability in number and composition of oral bacteria

Non-modifiable factors

Geographical location and culture have all been suggested to impact on oral bacteria composition. Findings from a study including participants from Northern and Southern Europe, reported a higher abundance of *Rothia* and unclassified *Gemellaceae* in Finnish populations compare to Spanish while *Lactococcus, Fusobacterium* and *Porphyromonas* genus were significantly higher in Spanish compare to Finnish groups[81]. Comparing findings of this study with another study which investigated the differences in oral bacteria between people living in Africa, Alaska and Germany showed that oral bacteria composition is highly variable between countries[82]. These differences may represent the sex and age distributions of these different populations, genetic make-up and habitual food preferences[83,82].

Moreover, the dorsal surface of the tongue plays a major role in nitrate reduction and represents a highly papillated surface area. The papillary structure of the human tongue is unique in nature and supports a higher bacterial density than the mucosal surface, accumulating oral debris and anaerobic bacteria on the rear of tongue[42]. There are three kinds of papillae on the tongue: fungiform, circumvallate and foliate papillae. The fungiform papillae have a mushroom shape and are found predominately on the dorsal surface of the tongue covering up to two-thirds of the surface. Their shape supports a higher bacterial density[84]. However, the shape and number of papillae varies between individuals which has been related to differences in oral bacteria composition. Studies have shown that a number of factors can affect the papillary number on the

tongue including ageing (with lower number of papillae observed in those individuals over 60 years), genetic make-up, ethnicity[81], demographics and environment[84].

Within the oral cavity, the presence of teeth increases the bacterial density compared to those with permanent tooth loss since the gingival crevices between teeth represent a greater surface area and environment for bacterial growth[85]. Other important factors considered to impact on the variety of nitrate reduction bacteria present in the oral cavity are ageing and sex. However, in a recent human study conducted in n=9 participants < 22 years and n=9 > 70 years, a similar salivary microbiome at baseline and after placebo beetroot juice was found in both groups. Comparable changes in bacterial composition (increases in *Rothia* and *Neisseria*) were also evident in both age groups in response to consuming 70 ml of beetroot juice (≈ 6.2 mmol nitrate) daily for 10 days[86] suggesting that age was not an important modulator of the oral bacteria composition in this study. Few studies have determined differences in oral bacteria composition between men and women. In order to address this knowledge gap, Kapil and colleagues[87] examined the impact of sex on nitrate reducing bacteria abundance in 13 male and 13 females age 18-45 years. Oral bacteria samples were collected before and after nitrate supplementation and all samples were analyzed by 16S rRNA sequencing. Significant sex dependent effects on oral nitrate reducing bacteria composition were not found in this study. However, sub-group analysis indicated females to have a non-significant tendency for a higher activity of nitrate reducing bacteria than men[87,74] but these findings need to be confirmed in a suitably powered study.

Modifiable factors

Several modifiable factors have been reported to influence and change the oral nitrate reducing bacteria composition, with dietary nitrate intake considered to be one of the most important factors[27,88]. In a recent cross-over study conducted in 18 volunteers assigned to receive a nitrate supplement or a placebo for 10 days, an increase in the abundance of some nitrate reducing bacteria, particularly *Rothia and Neisseria* was linked with the ability of an individual to reduce the nitrate

supplement. However, changes were not observed with the *Prevotella and Veillonella* species[86]. Interestingly, these results corroborate findings from another study which reported the reduction in *Prevotella* and *Veillonella* species in the oral cavity of elderly adults following dietary nitrate intake to be associated with a lower mortality risk in this population[88]. Furthermore, the increased prevalence of *Rothia* and *Neisseria* species relative to the *Prevotella* and *Veillonella* species was linked to higher NO bioavailability in both saliva and plasma[86]. These findings imply that the oral bacteria community is responsive to changes in the level of dietary nitrate intake[89]. However, the authors also reported that individuals with a higher abundance of *Campylobacter concisus* and *Prevotella melaninogenica* in their oral cavity at baseline may not be as responsive to dietary nitrate intake than those with a lower proportion of these bacteria[86]. This might reflect the fact that both *Campylobacter. concisus* and *Prevotella. melaninogenica* are predominately nitrite, but not nitrate, reducers in the oral cavity. Therefore, dietary nitrate availability may affect the growth and composition of particular groups of oral bacteria which can be related to improved cardiovascular health[89]. Of particular note, drinking beetroot juice rich in dietary nitrate can increase the oral cavity pH from 7.0 to 7.5 which is close to the optimal pH of 8 required for nitrate reductase activity[90]. Therefore, the effect of pH is also important in terms of the proliferation and inhibition of different populations within the oral bacterial community[86].

In a similar fashion, some health conditions have also been reported to influence the oral bacterial composition, with a lower density of nitrate reducing bacteria and a different bacterial composition found in people with raised blood pressure (hypertensives) than normotensive subjects[31]. A recent novel study has provided further evidence on the relationship between differences in oral bacteria composition with hypertension in postmenopausal women (n=446). This study analysed oral bacterial samples by using 16S RNA sequencing and found that the abundance of *Prevotella oral species 317* and *Streptococcus oralis* were significantly lower in women with elevated blood pressure compared with those with normal blood pressure[91]. Furthermore, the differences in the oral bacteria communities between groups also seemed to be associated with the

severity and progression of the hypertension [33]. Conversely, a higher abundance of nitrate reducing bacteria were observed in individuals who suffer from migraines (a vascular driven process associated with changes in NO). Interestingly, the dominant nitrate reducing bacteria in these individuals were *Pseudomonas* and *Streptococcus* which are not common in subjects who did not suffer with migraines. Oligotyping (the technique for differentiation between closely related microbial taxa)[92] was performed for both genera to investigate the strain-level differences across the bacterial population. *Pseudomonas* decompose to 2 oligotypes (different strains of the same species) and has differential abundance patterns with significantly higher abundance in oligotype 2 in those suffering from migraines compared with non-sufferers[93]. These results suggest that the type of these oral bacteria may be more prevalent in people with migraines. However, more work is needed to find the link and the mechanism to explain how these bacteria adapt genetically to their host environment.

Therefore, there may be an optimum number and composition of nitrate reducing bacteria which has beneficial effects, and a greater level may have a negative impact on conditions associated with blood vessel dilation such as migraine. However, it should be acknowledged that nitrate reduction and metabolism cannot be attributed to single bacterial species as they are unlikely to express all of the enzymes required to decompose nitrate simultaneously. More likely, these individual nitrate reducing bacteria are considered to work in synergy with other members of the microbial community. This has been demonstrated by Hyde et al, [43] who found that mixed colonies of high and low nitrate reducers showed a greater capacity for nitrate reduction than mixes of either multiple high reducers or individual nitrate reducing bacteria. This highlights the complexity of the oral microbiome and the impact on dietary nitrate metabolism.

Cardiometabolic diseases including obesity, the metabolic syndrome and type II diabetes are major contributors to global CVD disease burden. Whilst some studies have reported plasma nitrate/nitrite levels to be negatively associated with waist circumference [94], obesity [95] and blood pressure, others have observed positive associations between plasma nitrite and BMI, fasting blood

562 glucose [96], systolic blood pressure and the fasting lipid profile. In support of these findings, Akram
563 et al (2018)[97], found plasma nitrite levels to be higher in individuals with both obesity and the
564 metabolic syndrome followed by those with obesity alone, with the lowest levels in those with
565 normal weight. Whether high plasma nitrite levels play a role in the worsening of the
566 cardiometabolic risk markers is a public health issue since higher dietary nitrate intakes may also
567 cause higher levels of plasma NOx (sum of nitrate and nitrite levels). Furthermore, these data are
568 associations, do not indicate whether cardiometabolic risk markers change in response to varying
569 nitrate/nitrite intakes and do not prove cause and effect. Interestingly, a review of the evidence
570 suggests the contrary, with dietary nitrate supplementation found to reverse or improve some of the
571 features of the metabolic syndrome and be protective against the development of CVD[98]. Although
572 these beneficial effects may be related to improvements in NO metabolic pathways and glucose
573 control, we cannot discount that favourable changes in the gut microbiota in response to dietary
574 nitrate intake may also represent an important mechanism since dysbiosis (a term to describe
575 microbial imbalance) is a common feature of the cardiometabolic diseases. However, very few
576 studies have determined the impact of dietary nitrate supplementation on the gut microbiota in
577 humans, with a very short-term study with nitrate-rich fruit and vegetable juice suggesting a
578 reduction in the Firmicutes to Bacteroides ratio after 3 days which was related to higher plasma
579 nitrate/nitrite levels [99]. Furthermore, a one-year intervention with the Mediterranean diet, rich in
580 vegetables, was associated with increased abundance of specific taxa that were inversely associated
581 with inflammatory markers [100]. More studies are needed to address this research gap which also
582 include analysis of the oral microbiome to determine whether increases in the abundance of nitrate
583 reducing bacteria are related to improvements in cardiovascular health.

584 Oral hygiene habits, including daily use of an antibacterial mouthwash or tongue scraper
585 have been found to not only reduce acute bacterial infection, but also numbers of bacteria present[30].
586 On the other hand, poor oral hygiene contributes to dysbiosis by accumulating a plaque biofilm
587 which contains large number of microbes including nitrate reducing bacteria [101]. This can cause

588 dental infections and gingivitis by increasing pathogenic bacteria such as (*porphyromonas*
589 *gingivalis)*[102]. Studies have shown that patients with periodontal disease to have higher levels of
590 salivary nitrite which may be partly derived from the reduction of nitrates by the oral bacteria. Since
591 nitrite has been shown to have an antimicrobial effect against gastrointestinal and oral pathogens, it
592 has been speculated that the salivary glands may respond to the periodontal infection by enhancing
593 the secretion of nitrate and production of nitrite by the nitrate reducing bacteria as a host defence
594 mechanism[103]. This is thought to reduce the prevalence of the acidogenic bacteria which contribute
595 to the development of dental caries. In agreement, Doel et al [42] has reported a significant reduction
596 in dental carries in study participants with high salivary nitrate concentration. Epidemiological
597 studies have reported an association between periodontal disease with CVD. Although the cause
598 and effect relationship has not been proven, studies have suggested that inflammation caused by the
599 oral infection may contribute to the development and progression of the atherosclerotic plaque.
600 Interestingly, periodontal pathogens have been identified in the atherosclerotic plaque suggesting a
601 direct role in CVD. However, to date, periodontal disease has not been considered to be a CVD risk
602 marker[104]. Lifestyle habits such as smoking can also influence oral bacteria composition[74]. In a
603 study conducted in 9 non-smokers aged 20-45 y and n=5 healthy active smokers (>20 cigarettes per
604 week) aged 30-60 years, nitrate reduction activity was found to be over 80% lower in smokers
605 compared to non-smokers[74]. However, the low numbers of individuals within each group may have
606 influenced the results observed.

607 As previously mentioned, dietary nitrates have been shown to interact with other food
608 components such as lipids[72], with similar reports for polyphenols[105], alcohol[106] and proteins [25]. In
609 particular, foods and beverages rich in polyphenols including apple, tea and orange juice have been
610 shown to lead to a 3 fold increase in NO production in the stomach[107] and reduce endogenous N-
611 nitrosamine formation. Along with polyphenols, the content of ethanol in red wine can also interact
612 with nitrite forming ethyl nitrite which works as a nitrosation agent and may mediate NO effects.

These interactions with other dietary components may therefore play a role in modulating the circulating NO levels and bioavailability of the nitrate and nitrite contained within foods.

In summary, a systematic approach was used to identify the studies that have determined the impact of oral bacteria on blood pressure in response to nitrate intake, from dietary sources or supplements. However, only a very limited number of human (n=2) and animal (n=3) studies have addressed this research question, with the remaining studies examining the importance of the oral bacteria on the nitrate reducing capacity on circulating nitrite concentrations and blood pressure. Based on our observations from these studies, there is accumulating evidence to suggest that absence of nitrate-reducing oral bacteria was associated with increasing blood pressure even when accompanied by a high nitrate intake. However, some of the studies failed to see any effects, which may be due to type of mouthwash used in the human studies or the method of application of the mouthwash in the animal study[29]. Sex, hypertension, and tongue cleaning were all found to be important potential determinants of the variability in the responses between participants. Of these, the dental hygiene practice of tongue cleaning, which is recommended by the American Dental Association, appeared to promote oral microbiota diversity and be associated with a greater ability to recover the tongue microbiome after mouthwash use. Potential mechanisms to explain the blood pressure lowering effects of dietary nitrates included increases in plasma nitrite, S-nitrosothiols, nitro-fatty acids and vascular nitrosylation and cross-talk between the enterosalivary nitrate-nitrite-NO pathways and eNOS activity in the endothelial cells. However, the limited number of studies performed make it difficult to draw any firm conclusions from this literature review.

Conclusions

With the increasing prevalence of non-communicable diseases there is an urgent need for further studies to investigate the role of the oral bacteria on cardiovascular health in response to dietary nitrate intake, and to determine the underlying mechanisms. With vascular function now recognised as an important prognostic marker for future CVD risk, studies incorporating real time measures of

vascular reactivity and tone are required. Furthermore, the use of rigorous methods to determine changes in the abundance and composition of the oral bacteria in response to intake of dietary nitrate would help to identify important nitrate-reducing bacteria related to changes in vascular function and determine whether these bacterial groups are also evident in the gut microbiome, a proposed modulator of chronic disease risk. Diets containing nitrate-rich foods may contain other bioactive components which could also contribute to CVD risk reduction, including fibres, vitamins, minerals and flavonoids. Such diets may offer a number of advantages over nitrate/nitrite supplemental use, not only due to the availability of other bioactive components, but also because of reports of vascular adaptation and risk of marked acute hypotension after supplemental nitrate use, not found with nitrate-rich diets[108]. With hypertension a major risk factor for CVD, more studies are needed to determine whether diets higher in nitrate-rich foods can be recommended for blood pressure lowering and disease prevention in healthy individuals and those at greater CVD risk.

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Tables

Table1: Commonly reported nitrate reducing bacteria species found in the oral cavity

Bacteria species	Condition	Change in abundance in response to nitrate intake	Location in the oral cavity
Veillonella dispar[42,43]	Anaerobic	↑	Tongue
Actinomyces odontolyticus[42,43]	Facultative anaerobic	↑	Tongue
Prevotella salivae[42,43]	Anaerobic	↑	Tongue
Rothia mucilaginosa[42,14]	Aerobic	↑↑	Tongue
Neisseria flavescens[43,14]	Aerobic	↑↑	Tongue

Table 2: Animal studies investigating the importance of oral nitrate reducing bacteria on blood pressure in response to nitrate intake.

Reference	Animals	Study design and duration	Intervention	Measurement	Outcome measures
Petersson 2009[70]	n= 4-7 Male Sprague Dawley rats each group (190-360 g, B and K, Sollentwia, Sverge).	Parallel groups with 7 day treatment periods: 1) No treatment (control). 2) $NaNO_3$ only 3) Mouthwash 4) Mouthwash + $NaNO_3$ or $NaNO_2$	Water supplemented with 10 mM $NaNO_3$ or 1 mM $NaNO_2$ Mouthwash groups: Chlorhexidine mouthwash spray (0.3 ml), 2X daily.	Plasma	Δ NO_2 ↓ after mouthwash + $NaNO_3$ vs control $p<0.05$.
				HR SBP	NS NS
				DBP	↓after $NaNO_3$. DBP lowering absent in mouthwash treated rats
				MAP Oral bacteria	↓ after $NaNO_3$ and mouthwash + $NaNO_2$ vs mouthwash only. MAP lowering absent in mouthwash + $NaNO_3$ rats ↓ viable bacteria on tongue after mouthwash
Hyde 2014[29]	n= 8 Male Wistar rats 7 weeks old	19 day sequential intervention: 0-5 control (water) 6-12 $NaNO_3$, 13-19 $NaNO_3$ + mouthwash Blood collected at day 1, 5, 6, 12, 13 & 19. BP (telemetry) and tongue swab every day	$NaNO_3$ (1 g/L) in drinking water Mouthwash regime: 0.3 ml of chlorohexidine applied 2X daily to tongue dorsal surface (days 13-19)	SBP	NS
				DBP	↓ after $NaNO_3$ and mouthwash + $NaNO_3$ vs control
				Plasma NOx	NS

Pinheiro 2016[71]	n = 10, Male Wistar rats each group (190-210 g) 2 kidney, 1 clip (2K1C) hypertensive group. Sham operated control group	6 weeks – 2 weeks baseline followed by 4 weeks treatment Experiment 1 Vehicle $NaNO_2$ Mouthwash Mouthwash + $NaNO_2$ Experiment 2 Vehicle $NaNO_3$ Mouthwash Mouthwash + $NaNO_3$ 6 h after last treatment, blood and tongue swab collected.	15 mg $NaNO_2$/kg or 140 mg $NaNO_3$/kg (gavage) Mouthwash groups: Daily mouth clean with Chlorhexidine (0.12%) soaked swab.	Plasma	Δ NO_2 ↓ 25–30% after mouthwash **vs** $NaNO_2$ and $NaNO_3$ groups ($P < 0.05$) Δ NO_3 ↓ 45% after mouthwash vs $NaNO_2$ group ($P < 0.05$)
				BP	↓ SBP (40 mmHg) and MAP with $NaNO_2$ and $NaNO_3$ ($P= 0.01$). Mouthwash blunted MAP and SBP lowering effect of $NaNO_3$ ($p <0.05$) but not $NaNO_2$
				Oral bacteria	↓CFU 50-70% with mouthwash

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, NS: Not Significant, NO_2: Nitrite Concentration, SBP: Systolic Blood Pressure, NO_3: Nitrite Concentration, CFU: Colony Forming Unit (number of viable bacteria)

Table 3: Human studies determining the effects of oral bacteria on salivary and plasma nitrite concentrations, and/or blood pressure in response to nitrate intake.

Reference	Subject characteristics	Study design and duration	Nitrate dose	Type of mouthwash	Measurement	Significant outcomes
ACUTE STUDIES						
Mitsui et al., 2017 [75]	n=12 (6M/6F) Normotensive, Age 19-44 y Non-smoking,	Acute, RCT, CO 4 visits 10 h in duration with 1 wk washout. Saliva and oral bacteria collected 0, 1 and 10 h.	100 g lettuce (110 mg NO_3) with breakfast. Lunch at 5 h.	1. Water (control) 2. Listerine (antiseptic) 3. Isodine (povidone-iodine, 0.35%) 4. Chlorhexidine 0.0025% Treatment for 3 min prior to nitrate ingestion	Saliva Oral bacteria	Relative to baseline: ↑ NO_3 and NO_2 after each treatment ($P < 0.05$) ↓ nitrate reducing bacterium *V. Dispar* at 1 and 5 h after Chlorhexidine
Govoni et al 2008[27]	n=7 Normotensive Age 24-51y BMI 23 kg/m^2 Non-smoking	Acute, RCT, CO 2 visits of 3 h in duration. Blood and saliva samples collected before and for 3 h after nitrate intake. Oral bacteria collected in n=4 after mouthwash only.	10 mg/kg $NaNO_3$ in 100 ml water	Mouthwash vs no mouthwash Corsodyl (Chlorhexidine) gargled twice for 1 min, 15 min before nitrate ingestion.	Saliva Plasma Oral bacteria	↑NO_3 on both visits ↓ NO_2 vs no mouthwash NO_3 ↓ 29 nM and NO_2 ↓ 250 nM at 3 h vs no mouthwash ↓ bacteria count and (80%) and nitrate reducing capacity after mouthwash.

Woessner et al 2016[30]	n=12 (M) Normotensive $\overline{x}$ age 36 y and BMI 24 kg/m^2 Non-smoking	Acute, RCT, CO 4 visits, 4 h in duration with 1 wk washout. BP, blood and saliva collected before and for 4 h after juice consumption	140 ml of concentrated beetroot juice (8.4 mmol nitrate)	1) Water (control) 2) Listerine (antiseptic) 3) Cepacol (antibacterial) 4) Chlorhexidine (0.12%) Treatment 15 min after beetroot juice for 60s.	SBP	↓ Listerine and control vs Cepacol and Chlorhexidine ($P \leq 0.05$)
					DBP	NS
					Saliva	↑ NO_3 all treatments ↑ NO_2 control vs all mouthwashes and ↓ NO_2 Chlorhexidine and Cepacol vs antiseptic ($P \leq 0.05$)
					Plasma	↑ NO_3 all treatments ↓ NO_2 Chlorhexidine vs all treatments and Cepacol vs control ($P \leq 0.05$)

Bondonno et al 2012[76]	N=16 F Normotensive $\overline{x}$ age 52±11y (F) Non-smokers,	Acute, RCT, CO 5 visits of 3 h in duration. 1 wk washout. Blood and saliva samples collected before and for 3 h after nitrate intake	0, 100, 200, 400 mg NaNO3 in water	1) Antibacterial toothpaste (0.3% triclosan) 2) Toothpaste without antimicrobial agent (control)	Saliva Plasma	↑ NO_3 all treatments ↑ NO_3 all treatments
ACUTE WITHIN CHRONIC						
McDonagh et al 2016 [80]	n=12 (6M/6F) Normotensive $\overline{x}$ age 22±2y (F) and 24±2 y (M). Non-smokers,	Acute within chronic, RCT, double blind 6 visits over 8 weeks Each treatment 6 days, with acute visits (4 h) on days 0 and 6. Acute visits: Rinse with mouthwash 15 min before ingesting 2 x 70 ml beetroot juice. Measurements at 0, 2 and 4 h. BP and PWA measured at rest and during 10 min of treadmill walking. Saliva and plasma samples collected.	70 ml of beetroot juice (6.2mmol nitrate) twice a day	1) Strong - Corsodyl (Chlorhexidine) 2) Weak - Vademecum med (non-chlorhexidine-containing antibacterial mouthwash) 3) Deionised water (con) 3X daily 15 mins before beetroot juice and meals, for 6 days	SBP DBP MAP HR PWA	Relative to baseline (0 h): Resting - NS After 10 min exercise, ↑ 3 mmHg after strong mouthwash vs control (P = 0.07) 4 h after beetroot juice Resting and during exercise – NS Resting - NS After 10 min exercise, ↑ after strong mouthwash vs control (P<0.05) at 4 h. During exercise ↑ after strong vs control and weak (P<0.05). NS

					Plasma Saliva	↑NO_3 all treatments Δ NO2 ↓ after strong vs other treatments at 2 and 4 h, and weak vs control ($P<0.05$) at 2 h Δ NO3 ↑ and Δ NO2 ↓ after strong vs weak and control ($P<0.05$) at 4 h.

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, RCT: Randomized Controlled Trial, NS: Not Significant, PWA: Pulse Wave Analysis, SBP: Systolic Blood Pressure, CO: Cross Over.

Table 4: Chronic human studies investigating the involvement of oral bacteria in the blood pressure lowering effect of nitrate.

Reference	**Subject characteristics**	**Study design and duration**	**Oral nitrate reducing capacity**	**Mouthwash regime**	**Measurement**	**Significant outcome between treatment**
Tribble et al., 2019 [78]	n=26 (16F/10M) Normotensive Age 22-71 y	Sequential 4 visits over 14 days: days 1 (baseline), 7 (post mouth wash), 10 (recovery) and 14 (recovery) Clinic BP and oral bacteria at each visit. n=6 oral nitrate reducing capacity for 8 h after 30 s mouthwash	Mouth rinse with 1 mM $NaNO_3$ for 2 min	Chlorhexidine (0.12%) 2 x daily for 30 sec	SBP DBP Oral bacteria Oral nitrate reducing capacity	In response to mouthwash, ↑ 5mmHg (n=9) and ↓ (n=4) NS ↓ Species diversity and abundance with mouthwash for 7 days. ↑ bacterial metabolic activity at day 14. ↓ NO_3:NO_2 ratio for 6-8 h after mouthwash.

Sunqvist et al 2016 [79]	n=17 (F) Normotensive $\overline{x}$ age 23 y BMI = 22 kg/m^2 Non-smoking.	RCT, CO, double blind Each treatment 3 days with a 28 day washout 4 visits (days 3 and 4 of each treatment) 24 h ABP and urine sample. Clinic BP, saliva and plasma samples and oral nitrate reducing capacity	Mouth rinse with 10 mM $NaNO_3$ for 5 min	Chlorhexidine (0.2%) or placebo mouthwash 3 x daily after meals for 60s.	BP	No difference in ABP or clinic BP
					Saliva	↑ NO_3 and ↓ NO_2 after mouthwash ($P \leq 0.01$)
					Plasma Urine Oral nitrate reducing capacity	No change in NO_3 and NO_2 with mouthwash vs placebo excretion of NO_3 with mouthwash vs placebo ↓NO_2 after mouthwash (8 µM) vs placebo (234 µM)($P<0.001$)
Bondonno et al 2015 [77]	n=15 (8M/7F) Hypertensives taking medication	RCT, CO Each treatment 3 days with a 10-12 day washout.	Ratio of NO_2 and NO_3 measured in saliva.	Chlorhexidine or tap water (control)	SBP	↑ 2.3 mmHg after mouthwash vs water (P= 0.01)
					DBP	NS

	BP 120-159/100 mmHg. Age 53-69 y and BMI 20-35 kg/m^2. Non-smokers	Visits at day 0 and 3 of each treatment. Saliva sample, oral nitrate reducing capacity and plasma sample. BP measured at home.		2x daily with 20 ml for 30 sec after brushing teeth	Saliva Plasma Oral nitrate reducing capacity	↑ NO3 and ↓NO2 after mouthwash vs control (P= 0.001) ↓ NO2 after mouthwash vs control (P= 0.09). NO_3 - NS ↓ nitrate reductase ratio after mouthwash
Kapil et al 2013 [41]	n=19, Normotensive, Age 18-45y, BMI 18-40 kg/m^2, Non-smokers, No self-reported use of	Sequential 2 visits (0 and 14 days). At each visit, clinic BP, blood, urine and saliva samples and oral nitrate reduction capacity.	Mouth rinse after holding 3 doses of KNO_3 (0, 0.8 and 80 µmol) in the mouth for 5 min.	Chlorhexidine (0.2%) 2x daily days 8-14 only.	Clinic SBP	Relative to baseline, use of mouthwash ↑ 3.5mmHg (P = 0.003)
					Clinic DBP	↑ 2.2mmHg (P = 0.038)
					A-SBP A-DBP Home SBP Home DBP HR	↑2.4 mmHg (P= 0.017) ↑2.2 mmHg (P= 0.014) ↑2.9 mmHg (P< 0.001) ↑2.0 mmHg (P< 0.001) NS

	mouthwash or antibiotic	Fitted with ABP unit for 24 h and BP measured at home.			Saliva Plasma Urine Oral nitrate reducing capacity	↑NO_3 and ↓NO_2 90% (P< 0.001) ↑NO_3 and ↓NO_2 25% (P= 0.001) ↑NO_3 and ↓NO_2 At baseline, NO_2 in mouth rinse dose dependent (0<0.8<80 µmol KNO_3) After mouthwash, ↓ 90% NO_2 in mouth rinse for 0.8 and 80 µmol KNO_3.

Abbreviations: DBP: Diastolic Blood Pressure, HR: Heart Rate, MAP: Mean Arterial Pressure, RCT: Randomized Controlled Trial, NS: Not Significant, PWA: Pulse Wave Analysis, SBP: Systolic Blood Pressure, CO: Cross Over, ABP: Arterial Blood Pressure

FIGURE LEGENDS

Figure 1: Diagram of the endogenous generation of nitric oxide (NO) by NO synthase (NOS) (right panel highlighted in pink), and exogenous generation of NO from the diet (left panel highlighted in blue)[28]. In biological fluids, NO is oxidized to nitrite (NO_2) and nitrate (NO_3) (dashed arrows).

Figure 2: Overview of the nitrate enterosalivary circulation and nitrate metabolism in humans. Ingested inorganic nitrate is converted to nitrite in the oral cavity by nitrate reducing bacteria with reduction to NO and nitrogen oxides occurring within the acidic environment of the stomach. Remining nitrate and other nitrate components are then rapidly absorbed into the bloodstream via the small intestine. A large proportion of nitrate is then excreted by the kidneys into the urine, with up to 25% being recycled by the salivary glands and then concentrated in saliva.

Figure 3: Flow of information through the different phases of the literature review

FIGURE 1

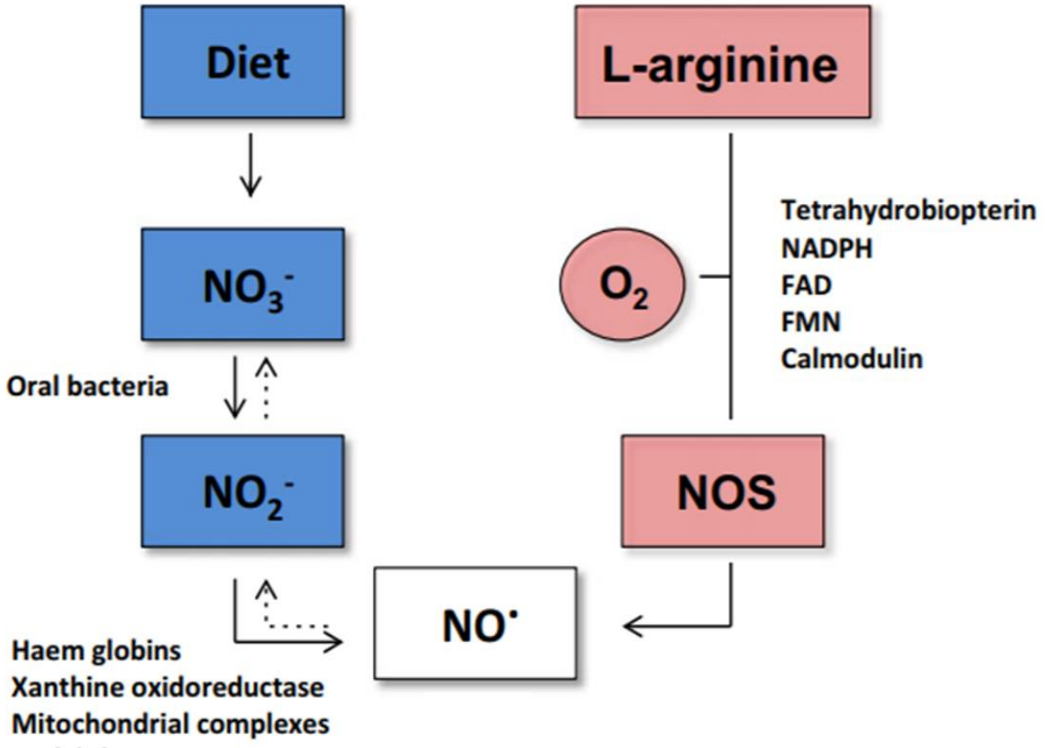

Diet
L-arginine
NO_3^-
O_2
Tetrahydrobiopterin
NADPH
FAD
FMN
Calmodulin
Oral bacteria
NO_2^-
NOS
NO·
Haem globins
Xanthine oxidoreductase
Mitochondrial complexes

FIGURE 2

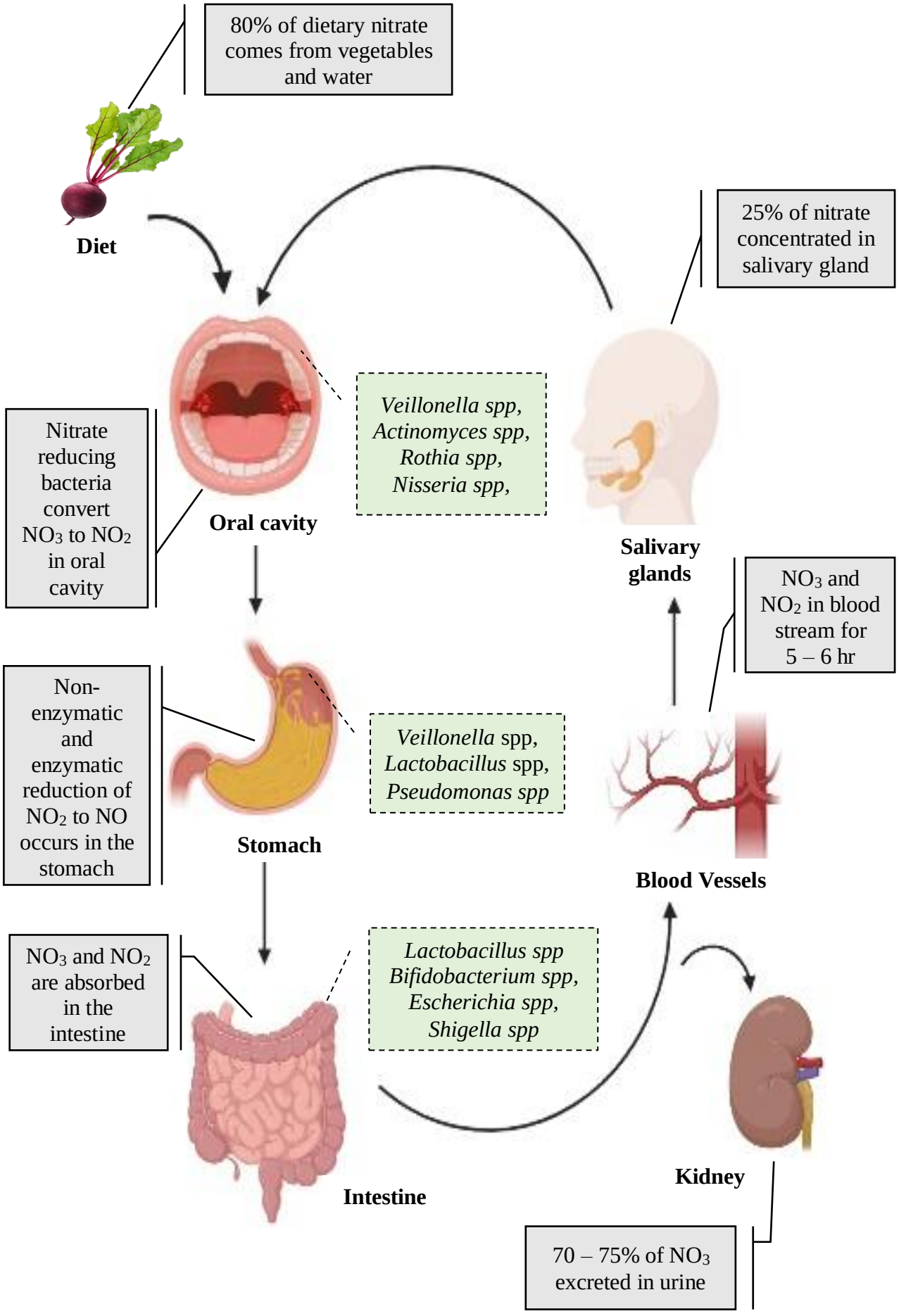
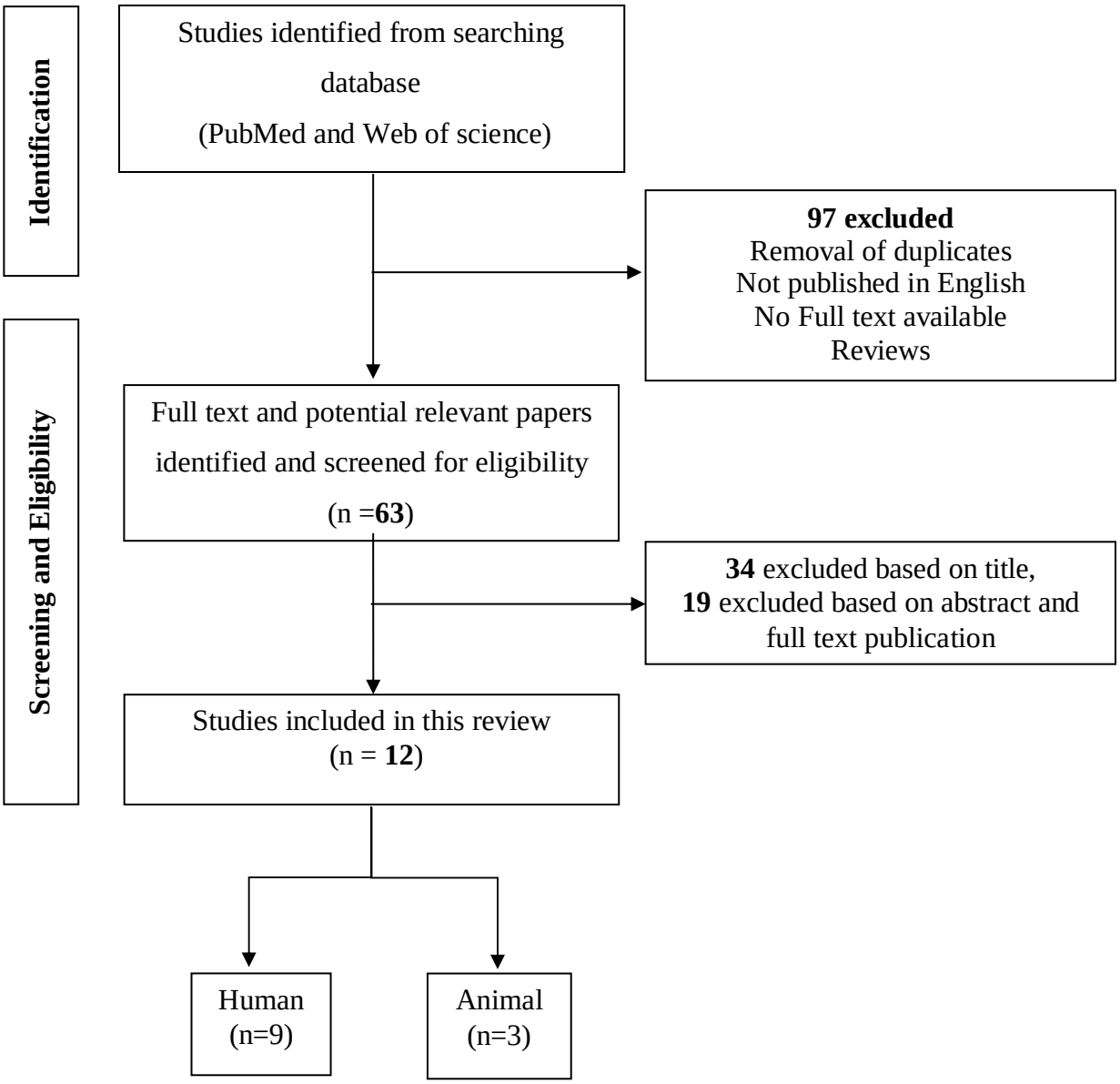

80% of dietary nitrate comes from vegetables and water
Diet
25% of nitrate concentrated in salivary gland
Veillonella spp, Actinomyces spp, Rothia spp, Nisseria spp,
Nitrate reducing bacteria convert NO3 to NO2 in oral cavity
Oral cavity
Salivary glands
NO3 and NO2 in blood stream for 5 – 6 hr
Non-enzymatic and enzymatic reduction of NO2 to NO occurs in the stomach
Veillonella spp, Lactobacillus spp, Pseudomonas spp
Stomach
Blood Vessels
NO3 and NO2 are absorbed in the intestine
Lactobacillus spp Bifidobacterium spp, Escherichia spp, Shigella spp
Intestine
Kidney
70 – 75% of NO3 excreted in urine

FIGURE 3


Identification
Studies identified from searching database (PubMed and Web of science)
97 excluded
Removal of duplicates
Not published in English
No Full text available
Reviews
Screening and Eligibility
Full text and potential relevant papers identified and screened for eligibility (n =63)
34 excluded based on title,
19 excluded based on abstract and full text publication
Studies included in this review (n = 12)
Human (n=9)
Animal (n=3)